

COPY

TITLE OF THE INVENTION

Nucleotide sequences which code for the metY gene

BACKGROUND OF THE INVENTION

5 Field of the Invention

The invention provides nucleotide sequences from coryneform bacteria which code for the metY gene and a process for the fermentative preparation of amino acids, in particular L-lysine and L-methionine, using bacteria in which at least the metY gene is enhanced.

10 Description of the Related Art

L-Amino acids, in particular L-lysine and L-methionine, are used in human medicine and in the pharmaceuticals of industry, in the foodstuffs industry and very particularly in animal nutrition.

15 It is known that amino acids are prepared by fermentation from strains of coryneform bacteria, in particular *Corynebacterium glutamicum*. Because of their great importance, work is constantly being undertaken to improve the preparation process. Improvements to the process can relate to

20 fermentation measures stirring and supply of oxygen, or to the composition of the nutrient media, such as the sugar concentration during the fermentation, or to working up of the product by, for example, ion exchange chromatography, or to

25 the intrinsic output properties of the microorganism itself.

30 Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites or are auxotrophic for metabolites of regulatory importance and produce amino acids are obtained in this manner.

Recombinant DNA techniques have also been employed for some years for improving the *Corynebacterium* strains which produce

L-amino acid, by amplifying individual amino acid biosynthesis genes and investigating their effect on amino acid production.

SUMMARY OF THE INVENTION

An object of the present invention is to provide new measures 5 for improved fermentative preparation of amino acids, in particular L-lysine and L-methionine.

Where L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine.

When L-lysine or lysine are mentioned in the following, not 15 only the bases but also the salts, such as lysine monohydrochloride or lysine sulfate, are intended.

When L-methionine or methionine are mentioned in the following, the salts, such as e.g. methionine hydrochloride or methionine sulfate, are intended.

20 The invention provides an isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence which codes for the metY gene, chosen from the group consisting of

- a) polynucleotide which is at least 70% identical to a 25 polynucleotide that codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,
- b) polynucleotide which codes for a polypeptide that comprises an amino acid sequence which is at least 70% identical to the amino acid sequence of SEQ ID No. 2,
- 30 c) polynucleotide which is complementary to the polynucleotides of a) or b), and

d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

and the corresponding polypeptides having the enzymatic activity of O-acetylhomoserine sulfhydrylase.

5 The invention also provides the above-mentioned polynucleotides as DNA which is capable of replication, comprising:

(i) the nucleotide sequence shown in SEQ ID No. 1, or

(ii) at least one sequence which corresponds to sequence
10 (i) within the range of the degeneration of the genetic code, or

(iii) at least one sequence which hybridizes with the sequence complementary to sequence (i) or (ii), and optionally

15 (iv) sense mutations of neutral function in (i).

The invention also provides

a polynucleotide comprising the nucleotide sequence as shown in SEQ ID No. 1;

20 a polynucleotide that codes for a polypeptide which comprises the amino acid sequence as shown in SEQ ID No. 2;

a vector containing the DNA sequence of *C. glutamicum* that codes for the *metY* gene, deposited in accordance with the Budapest Treaty in *Corynebacterium glutamicum* as pCREmetY on June 06, 2000 under DSM 13556

25 and coryneform bacteria in which the *metY* gene is present in enhanced form, in particular by the vector pCREmetY.

The invention also provides polynucleotides which are obtained by screening a corresponding gene library of a coryneform bacterium, which comprises the complete gene or parts thereof, 30 by means of hybridization with a probe which comprises the

sequence of the polynucleotide according to the invention according to SEQ ID No.1 or a fragment thereof, and isolation of the polynucleotide sequence mentioned.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Fig. 1 shows plasmid pCREmetY.

Fig. 2 shows plasmid pCREmetAY.

Detailed Description of the Preferred Embodiments

Polynucleotides which comprise the sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate, in the full length, nucleic acids or polynucleotides or genes which code for O-acetylhomoserine sulfhydrylase or to isolate those nucleic acids or polynucleotides or genes which have a high similarity of sequence or homology with that of the O-acetylhomoserine sulfhydrylase.

Polynucleotides according to the invention are furthermore suitable as primers with which DNA of genes that code for O-acetylhomoserine sulfhydrylase can be prepared by the polymerase chain reaction (PCR).

20 Such oligonucleotides that serve as probes or primers comprise at least 30, preferably at least 20, very particularly at least 15 successive nucleotides. Oligonucleotides which have a length of at least 40 or 50 nucleotides are also suitable. Oligonucleotides with a length of at least 100, 150, 200, 250
25 or 300 nucleotides are optionally also suitable.

"Isolated" means separated out of its natural environment.

"Polynucleotide" in general relates to polyribonucleotides and polydeoxyribonucleotides, it being possible for these to be non-modified RNA or DNA or modified RNA or DNA.

30 The polynucleotides according to the invention include a polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom and also those which are at least 70%,

preferably at least 80% and in particular at least 90% to 95% identical to the polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom.

"Polypeptides" are understood as meaning peptides or proteins
5 which comprise two or more amino acids bonded via peptide bonds.

The polypeptides according to the invention include a polypeptide according to SEQ ID No. 2, in particular those with the biological activity of O-acetylhomoserine
10 sulfhydrylase, and also those which are at least 70%, preferably at least 80%, and in particular which are at least 90% to 95% identical to the polypeptide according to SEQ ID No. 2 and have the activity mentioned.

The invention moreover provides a process for the fermentative
15 preparation of amino acids, in particular L-lysine and L-methionine, using coryneform bacteria which in particular already produce amino acids, and in which at least the nucleotide sequences which code for the metY gene are enhanced, in particular over-expressed.

20 The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or using a gene which codes for 25 a corresponding enzyme having a high activity, and optionally combining these measures.

By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%,
30 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on the starting microorganism.

The microorganisms which the present invention provides can prepare L-amino acids, in particular L-lysine and L-methionine, from glucose, sucrose, lactose, fructose, maltose,

molasses, starch, cellulose or from glycerol and ethanol. They can be representatives of coryneform bacteria, in particular of the genus *Corynebacterium*. Of the genus *Corynebacterium*, there may be mentioned in particular the species *Corynebacterium glutamicum*, which is known among experts for its ability to produce L-amino acids.

Suitable strains of the genus *Corynebacterium*, in particular of the species *Corynebacterium glutamicum* (*C. glutamicum*), are in particular the known wild-type strains

10 *Corynebacterium glutamicum* ATCC13032
 Corynebacterium acetoglutamicum ATCC15806
 Corynebacterium acetoacidophilum ATCC13870
 Corynebacterium thermoaminogenes FERM BP-1539
 Corynebacterium melassecola ATCC17965
15 *Brevibacterium flavum* ATCC14067
 Brevibacterium lactofermentum ATCC13869 and
 Brevibacterium divaricatum ATCC14020

or L-lysine-producing mutants or strains prepared therefrom, such as, for example

20 *Corynebacterium glutamicum* FERM-P 1709
 Brevibacterium flavum FERM-P 1708
 Brevibacterium lactofermentum FERM-P 1712
 Corynebacterium glutamicum FERM-P 6463
 Corynebacterium glutamicum FERM-P 6464 and
25 *Corynebacterium glutamicum* DSM5715.

or L-methionine-producing mutants or strains prepared therefrom, such as, for example

Corynebacterium glutamicum ATCC21608.

The new *metY* gene from *C. glutamicum* which codes for the 30 enzyme O-acetylhomoserine sulphhydrylase (EC 4.2.99.10) has been isolated.

To isolate the *metY* gene or also other genes of *C. glutamicum*, a gene library of this microorganism is first set up in

Escherichia coli (E. coli). The setting up of gene libraries is described in generally known textbooks and handbooks. The textbook by Winnacker: Gene und Klone, Eine Einführung in die Gentechnologie (Verlag Chemie, Weinheim, Germany, 1990), or the handbook by Sambrook et al.: Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989) may be mentioned as an example. A well-known gene library is that of the E. coli K-12 strain W3110 set up in λ vectors by Kohara et al. (Cell 50, 495 -508 (1987)). Bathe et al. (Molecular and General Genetics, 252:255-265, 1996) describe a gene library of C. glutamicum ATCC13032, which was set up with the aid of the cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA, 84:2160-2164) in the E. coli K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575).

Börmann et al. (Molecular Microbiology 6(3), 317-326)) (1992)) in turn describe a gene library of C. glutamicum ATCC13032 using the cosmid pHCT79 (Hohn and Collins, Gene 11, 291-298 (1980)). To prepare a gene library of C. glutamicum in E.

coli it is also possible to use plasmids such as pBR322 (Bolivar, Life Sciences, 25, 807-818 (1979)) or pUC9 (Vieira et al., 1982, Gene, 19:259-268). Suitable hosts are, in particular, those E. coli strains which are restriction- and recombination-defective. An example of these is the strain DH5 α mcr, which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649). The long DNA fragments cloned with the aid of cosmids can in turn be subcloned in the usual vectors suitable for sequencing and then sequenced, as is described e.g. by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 1977).

The resulting DNA sequences can then be investigated with known algorithms or sequence analysis programs, such as that of Staden (Nucleic Acids Research 14, 217-232(1986)), that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)).

The new DNA sequence of *C. glutamicum* which codes for the metY gene and which, as SEQ ID No. 1, is a constituent of the present invention has been found. The amino acid sequence of the corresponding protein has furthermore been derived from 5 the present DNA sequence by the methods described above. The resulting amino acid sequence of the metY gene product is shown in SEQ ID No. 2.

Coding DNA sequences which result from SEQ ID No. 1 by the degeneracy of the genetic code are also a constituent of the 10 invention. In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Conservative amino acid exchanges, such as e.g. exchange of glycine for alanine or of aspartic acid for glutamic acid in proteins, are furthermore known among experts 15 as "sense mutations" which do not lead to a fundamental change in the activity of the protein, i.e. they are of neutral function.

It is furthermore known that changes at the N and/or C terminus of a protein must not substantially impair or may 20 even stabilize the function thereof. Information in this context can be found in Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)), in O'Regan et al. (Gene 77:237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)), in Hochuli et al. (Bio/Technology 6:1321- 25 1325 (1988)) and in known textbooks of genetics and molecular biology. Amino acid sequences which result in a corresponding manner from SEQ ID No. 2 are also a constituent of the invention.

In the same way, DNA sequences which hybridize with SEQ ID No. 30 1 or parts of SEQ ID No. 1 are a constituent of the invention. Finally, DNA sequences which are prepared by the polymerase chain reaction (PCR) using primers which result from SEQ ID No. 1 are a constituent of the invention. Such oligonucleotides typically have a length of at least 15 35 nucleotides.

Instructions for identifying DNA sequences by means of hybridization can be found in the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al.

5 (International Journal of Systematic Bacteriology (1991), 41: 255-260). The hybridization takes place under stringent conditions, that is to say only hybrids are formed in which the probe and target sequence, i.e. the polynucleotides treated with the probe, are at least 70% identical. It is
10 known that the stringency of the hybridization, including the washing steps, is influenced or determined by varying the buffer composition, the temperature and the salt concentration. The hybridization reaction is preferably carried out under a relatively low stringency compared with
15 the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996).

A 5x SSC buffer at a temperature of approx. 50 - 68°C, for example, can be employed for the hybridization reaction. Probes can also hybridize here with polynucleotides which are
20 less than 70% identical to the sequence of the probe. Such hybrids are less stable and are removed by washing under stringent conditions. This can be achieved, for example, by lowering the salt concentration to 2x SSC and optionally subsequently 0.5x SSC (The DIG System User's Guide for Filter
25 Hybridisation, Boehringer Mannheim, Mannheim, Germany, 1995) a temperature of approx. 50 - 68°C being established. It is optionally possible to lower the salt concentration to 0.1x SSC. Polynucleotide fragments which are, for example, at least 70% or at least 80% or at least 90% to 95% identical to
30 the sequence of the probe employed can be isolated by increasing the hybridization temperature stepwise from 50 to 68°C in steps of approx. 1 - 2°C. Further instructions on hybridization are obtainable on the market in the form of so-called kits (e.g. DIG Easy Hyb from Roche Diagnostics GmbH,
35 Mannheim, Germany, Catalogue No. 1603558).

Instructions for amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) can be found in the

handbook by Gait: Oligonucleotide synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

5 It has been found that coryneform bacteria produce amino acids, in particular L-lysine and L-methionine, in an improved manner after over-expression of the metY gene, optionally in combination with the metA gene.

To achieve an over-expression, the number of copies of the 10 corresponding genes can be increased, or the promoter and regulation region or the ribosome binding site upstream of the structural gene can be mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is additionally possible 15 to increase the expression in the course of fermentative L-lysine and L-methionine production. The expression is likewise improved by measures to prolong the life of the mRNA. Furthermore, the enzyme activity is also increased by preventing the degradation of the enzyme protein. The genes 20 or gene constructs can either be present in plasmids with a varying number of copies, or can be integrated and amplified in the chromosome. Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

25 Instructions in this context can be found in Martin et al. (Bio/Technology 5, 137-146 (1987)), in Guerrero et al. (Gene 138, 35-41 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), in Eikmanns et al. (Gene 102, 93-98 (1991)), 30 in European Patent Specification 0 472 869, in US Patent 4,601,893, in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)), in Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)), in LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)), in Patent Application WO 96/15246, in Malumbres et al. (Gene 134, 15 - 24 (1993)), 35 in Japanese Laid-Open Specification JP-A-10-229891, in Jensen and Hammer (Biotechnology and Bioengineering 58, 191-195

(1998)), in Makrides (Microbiological Reviews 60:512-538 (1996)) and in known textbooks of genetics and molecular biology.

By way of example, for enhancement the metY gene according to
5 the invention was over-expressed with the aid of episomal plasmids. Suitable plasmids are those which are replicated in coryneform bacteria. Numerous known plasmid vectors, such as e.g. pZ1 (Menkel et al., Applied and Environmental Microbiology (1989) 64: 549-554), pEKEx1 (Eikmanns et al.,
10 Gene 102:93-98 (1991)) or pHs2-1 (Sonnen et al., Gene 107:69-74 (1991)) are based on the cryptic plasmids pHM1519, pBL1 or pGA1. Other plasmid vectors, such as those based on pCG4 (US-A 4,489,160), or pNG2 (Serwold-Davis et al., FEMS Microbiology Letters 66, 119-124 (1990)), or pAG1 (US-A 5,158,891), can be
15 used in the same manner.

Examples of such plasmid vectors are shown in figures 1 and 2.

Plasmid vectors which are furthermore suitable are also those with the aid of which the process of gene amplification by integration into the chromosome can be used, as has been
20 described, for example, by Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)) for duplication or amplification of the hom-thrB operon. In this method, the complete gene is cloned in a plasmid vector which can replicate in a host (typically E. coli), but not in C.
25 glutamicum. Possible vectors are, for example, pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pGEM-T (Promega corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994), Journal of Biological Chemistry 269:32678-84; US-A 5,487,993),
30 pCR®Blunt (Invitrogen, Groningen, Holland; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)), pEM1 (Schrumpf et al., 1991, Journal of Bacteriology 173:4510-4516) or pBGS8 (Spratt et al., 1986, Gene 41: 337-342). The plasmid vector which contains the gene to be amplified is then
35 transferred into the desired strain of C. glutamicum by conjugation or transformation. The method of conjugation is

described, for example, by Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)). Methods for transformation are described, for example, by Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), 5 Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of a "cross over" event, the resulting strain contains at least two copies of the gene in question.

10 In addition, it may be advantageous for the production of amino acids, in particular L-lysine and L-methionine, to enhance one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, or of amino acid export, in addition to the metY gene.

15 Thus, for example, for the preparation of L-lysine one or more genes chosen from the group consisting of

- the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- 20 • the tpi gene which codes for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the pgk gene which codes for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- 25 • the pyc gene which codes for pyruvate carboxylase (DE-A-198 31 609),
- the lysC gene which codes for a feed-back resistant aspartate kinase (ACCESSION NUMBER P26512),

can be enhanced, in particular over-expressed.

Thus, for example, for the preparation of L-methionine one or 30 more genes chosen from the group consisting of

- the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
 - the tpi gene which codes for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
 - the pgk gene which codes for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
 - the pyc gene which codes for pyruvate carboxylase (DE-A-198 31 609),
 - the lysC gene which codes for a feed-back resistant aspartate kinase (ACCESSION NUMBER P26512),
 - the metA gene which codes for homoserine O-acetyltransferase (ACCESSION Number AF052652),
 - the metB gene which codes for cystathionine-gamma-synthase (ACCESSION Number AF126953),
 - the aecD gene which codes for cystathionine-gamma-lyase (ACCESSION Number M89931)
 - the glyA gene which codes for serine hydroxymethyltransferase (JP-A-08107788),
- can be enhanced, in particular over-expressed, additional enhancement of metA being particularly preferred.

It may furthermore be advantageous for the production of L-lysine, in addition to the enhancement of the metY gene, for one or more genes chosen from the group consisting of

- the pck gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1; DSM 13047),
- the pgi gene which codes for glucose 6-phosphate isomerase (US 09/396,478, DSM 12969),
- the poxB gene which codes for pyruvate oxidase (DE: 1995 1975.7; DSM 13114)

to be attenuated, in particular for the expression thereof to be reduced.

It may furthermore be advantageous for the production of L-methionine, in addition to the enhancement of the metY gene,
5 for one or more genes chosen from the group consisting of

- the pck gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1; DSM 13047),
 - the pgi gene which codes for glucose 6-phosphate isomerase (US 09/396,478, DSM 12969),
 - 10 • the poxB gene which codes for pyruvate oxidase (DE: 1995 1975.7; DSM 13114)
 - the thrB gene which codes for homoserine kinase (ACCESSION Number P08210),
 - 15 • the ilvA gene which codes for threonine dehydratase (ACCESSION Number Q04513),
 - the thrC gene which codes for threonine synthase (ACCESSION Number P23669),
 - the ddh gene which codes for meso-diaminopimelate D-dehydrogenase (ACCESSION Number Y00151),
- 20 to be attenuated, in particular for the expression thereof to be reduced.

The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded
25 by the corresponding DNA, for example by using a weak promoter or using a gene or allele which codes for a corresponding enzyme with a low activity or inactivates the corresponding gene or enzyme (protein), and optionally combining these measures.

30 By attenuation measures, the activity or concentration of the corresponding protein is in general reduced to 0 to 50%, 0 to

25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein.

In addition to over-expression of the metY gene, optionally in combination with the metA gene it may furthermore be
5 advantageous for the production of amino acids, in particular L-lysine and L-methionine, to eliminate undesirable side reactions, (Nakayama: "Breeding of Amino Acid Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK,
10 1982).

The microorganisms prepared according to the invention can be cultured continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for the purpose of
15 production of amino acids, in particular L-lysine and L-methionine. A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und
20 periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the
25 handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose,
30 oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substance
35 can be used individually or as a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium

5 carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing

10 salts can be used as the source of phosphorus.

Organic and inorganic sulfur-containing compounds, such as, for example, sulfides, sulfites, sulfates and thiosulfates, can be used as a source of sulfur, in particular for the preparation of sulfur-containing amino acids.

15 The culture medium must furthermore comprise salts of metals, such as e. g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the above-mentioned substances. Suitable precursors can 20 moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium 25 hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective 30 action, such as e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 35 40°C. Culturing is continued until a maximum of the desired

product has formed. This target is usually reached within 10 hours to 160 hours.

The fermentation broths obtained in this way, in particular containing L-methionine, usually have a dry weight of 7.5 to 5 25 wt.% and contain L-methionine. It is furthermore also advantageous if the fermentation is conducted in a sugar-limited procedure at least at the end, but in particular over 10 at least 30% of the duration of the fermentation. That is to say, the concentration of utilizable sugar in the fermentation medium is reduced to ≥ 0 to 3 g/l during this period.

The fermentation broth prepared in this manner, in particular containing L-methionine, is then further processed. Depending on requirements all or some of the biomass can be removed from 15 the fermentation broth by separation methods, such as centrifugation, filtration, decanting or a combination thereof, or it can be left completely in. This broth is then thickened or concentrated by known methods, such as with the aid of a rotary evaporator, thin film evaporator, falling film evaporator, by reverse osmosis, or by nanofiltration. This 20 concentrated fermentation broth can then be worked up by methods of freeze drying, spray drying, spray granulation or by other processes to give a preferably free-flowing, finely divided powder.

This free-flowing, finely divided powder can then in turn by 25 converted by suitable compacting or granulating processes into a coarse-grained, readily free-flowing, storable and largely dust-free product. In the granulation or compacting it is advantageous to employ conventional organic or inorganic auxiliary substances or carriers, such as starch, gelatin, 30 cellulose derivatives or similar substances, such as are conventionally used as binders, gelling agents or thickeners in foodstuffs or feedstuffs processing, or further substances, such as, for example, silicas, silicates or stearates.

"Free-flowing" is understood as meaning powders which flow 35 unimpeded out of the vessel with the opening of 5 mm (millimeters) of a series of glass outflow vessels with

outflow openings of various sizes (Klein, Seifen, Öle, Fette, Wachse 94, 12 (1968)).

As described here, "finely divided" means a powder with a predominant content (> 50 %) having a particle size of 20 to 5 200 µm diameter. "Coarse-grained" means products with a predominant content (> 50 %) having a particle size of 200 to 2000 µm diameter. In this context, "dust-free" means that the product contains only small contents (< 5 %) having particle sizes of less than 20 µm diameter. The particle size 10 determination can be carried out with methods of laser diffraction spectrometry. The corresponding methods are described in the textbook on "Teilchengrößenmessung in der Laborpraxis" by R. H. Müller and R. Schuhmann, 15 Wissenschaftliche Verlagsgesellschaft Stuttgart (1996) or in the textbook "Introduction to Particle Technology" by M. Rhodes, Verlag Wiley & Sons (1998).

"Storable" in the context of this invention means a product which can be stored for up to 120 days, preferably up to 52 weeks, particularly preferably 60 months, without a 20 substantial loss (< 5%) of methionine occurring.

Alternatively, however, the product can be absorbed on to an organic or inorganic carrier substance which is known and conventional in feedstuffs processing, for example, silicas, silicates, grits, brans, meals, starches, sugars or others, 25 and/or mixed and stabilized with conventional thickeners or binders. Use examples and processes in this context are described in the literature (Die Mühle + Mischfuttertechnik 132 (1995) 49, page 817).

Finally, the product can be brought into a state in which it 30 is stable to digestion by animal stomachs, in particular the stomach of ruminants, by coating processes ("coating") using film-forming agents, such as, for example, metal carbonates, silicas, silicates, alginates, stearates, starches, gums and cellulose ethers, as described in DE-C-4100920.

If the biomass is separated off during the process, further inorganic solids, for example added during the fermentation, are in general removed. In addition, the animal feedstuffs additive according to the invention comprises at least the predominant proportion of the further substances, in particular organic substances, which are formed or added and are present in solution in the fermentation broth, where these have not been separated off by suitable processes.

In one aspect of the invention, the biomass can be separated off to the extent of up to 70%, preferably up to 80%, preferably up to 90%, preferably up to 95%, and particularly preferably up to 100%. In another aspect of the invention, up to 20% of the biomass, preferably up to 15%, preferably up to 10%, preferably up to 5%, particularly preferably no biomass is separated off.

These organic substances include organic by-products which are optionally produced, in addition to the L-methionine, and optionally discharged by the microorganisms employed in the fermentation. These include L-amino acids chosen from the group consisting of L-lysine, L-valine, L-threonine, L-alanine or L-tryptophan. They include vitamins chosen from the group consisting of vitamin B1 (thiamine), vitamin B2 (riboflavin), vitamin B5 (pantothenic acid), vitamin B6 (pyridoxine), vitamin B12 (cyanocobalamin), nicotinic acid/nicotinamide and vitamin E (tocopherol). They also include organic acids which carry one to three carboxyl groups, such as, acetic acid, lactic acid, citric acid, malic acid or fumaric acid. Finally, they also include sugars, for example, trehalose. These compounds are optionally desired if they improve the nutritional value of the product.

These organic substances, including L-methionine and/or D-methionine and/or the racemic mixture D,L-methionine, can also be added, depending on requirements, as a concentrate or pure substance in solid or liquid form during a suitable process step. These organic substances mentioned can be added individually or as mixtures to the resulting or concentrated

fermentation broth, or also during the drying or granulation process. It is likewise possible to add an organic substance or a mixture of several organic substances to the fermentation broth and a further organic substance or a further mixture of several organic substances during a later process step, for example granulation.

The product described above is suitable as a feedstuffs additive, i.e. feed additive, for animal nutrition.

The L-methionine content of the animal feedstuffs additive is conventionally 1 wt.% to 80 wt.%, preferably 2 wt.% to 80 wt.%, particularly preferably 4 wt.% to 80 wt.%, and very particularly preferably 8 wt.% to 80 wt.%, based on the dry weight of the animal feedstuffs additive. Contents of 1 wt.% to 60 wt.%, 2 wt.% to 60 wt.%, 4 wt.% to 60 wt.%, 6 wt.% to 60 wt.%, 1 wt.% to 40 wt.%, 2 wt.% to 40 wt.% or 4 wt.% to 40 wt.% are likewise possible. The water content of the feedstuffs additive is conventionally up to 5 wt.%, preferably up to 4 wt.%, and particularly preferably less than 2 wt.%.

The invention also provides a process for the preparation of an L-methionine-containing animal feedstuffs additive from fermentation broths, which comprises the steps

- a) culture and fermentation of an L-methionine-producing microorganism in a fermentation medium;
- b) removal of water from the L-methionine-containing fermentation broth (concentration);
- c) removal of an amount of 0 to 100 wt.% of the biomass formed during the fermentation; and
- d) drying of the fermentation broth obtained according to a) and/or b) to obtain the animal feedstuffs additive in the desired powder or granule form.

If desired, one or more of the following steps can furthermore be carried out in the process according to the invention:

e) addition of one or more organic substances, including L-methionine and/or D-methionine and/or the racemic mixture D,L-methionine, to the products obtained according to a), b) and/or c);

5 f) addition of auxiliary substances chosen from the group consisting of silicas, silicates, stearates, grits and bran to the substances obtained according to a) to d) for stabilization and to increase the storability; or

10 g) conversion of the substances obtained according to a) to e) into a form which remains stable in an animal stomach, in particular rumen, by coating with film-forming agents.

The analysis of L-lysine and L-methionine can be carried out by ion exchange chromatography with subsequent ninhydrin derivation, as described by Spackman et al. (Analytical 15 Chemistry, 30, (1958), 1190).

The following microorganism was deposited as a pure culture on June 21, 2000 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the 20 Budapest Treaty:

- Corynebacterium glutamicum strain DSM5715/pCREmetY as DSM 13556

The process according to the invention is used for the fermentative preparation of amino acids, in particular L-lysine and L-methionine. 25

The present invention is explained in more detail in the following with the aid of embodiment examples.

Example 1

Preparation of a genomic cosmid gene library from 30 Corynebacterium glutamicum ATCC 13032

Chromosomal DNA from Corynebacterium glutamicum ATCC 13032 was isolated as described by Tauch et al. (1995, Plasmid 33:168-

179) and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Code no. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987), Proceedings of the National Academy of Sciences, USA, 84:2160-2164), obtained from Stratagene (La Jolla, USA, Product Description SuperCos1 Cosmid Vector Kit, Code no. 251301) was cleaved with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, Product Description XbaI, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase.

The cosmid DNA was then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Code no. 27-0868-04). The cosmid DNA treated in this manner was mixed with the treated ATCC13032 DNA and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no. 27-0870-04). The ligation mixture was then packed in phages with the aid of Gigapack II XL Packing Extract (Stratagene, La Jolla, USA, Product Description Gigapack II XL Packing Extract, Code no. 200217).

For infection of the E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Research 16:1563-1575) the cells were taken up in 10 mM MgSO₄ and mixed with an aliquot of the phage suspension. The infection and titering of the cosmid library were carried out as described by Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the cells being plated out on LB agar (Lennox, 1955, Virology, 1:190) with 100 mg/l ampicillin. After incubation overnight at 37°C, recombinant individual clones were selected.

Example 2

Isolation and sequencing of the metY gene

The cosmid DNA of an individual colony was isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions

5 and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Product No. 27-0913-02). The DNA fragments were

dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, the cosmid fragments in the size range of

10 1500 to 2000 bp were isolated with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

15 The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, The Netherlands, Product Description Zero Background Cloning Kit, Product No. K2500-01) was cleaved with the restriction enzyme BamHI (Amersham Pharmacia,

Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04). The ligation of the cosmid fragments in the

20 sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This

25 ligation mixture was then electroporated (Tauch et al. 1994, FEMS Microbiol. Letters, 123:343-7) into the E. coli strain DH5 α mcr (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649) and plated out on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l zeocin.

30 The plasmid preparation of the recombinant clones was carried out with Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). The sequencing was carried out by the dideoxy chain termination method of Sanger et al. (1977, Proceedings of the

35 National Academy of Sciences U.S.A., 74:5463-5467) with modifications according to Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The "RR dRhodamin Terminator Cycle

Sequencing Kit" from PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany) was used. The separation by gel electrophoresis and analysis of the sequencing reaction were carried out in a "Rotiphoresis NF Acrylamide/Bisacrylamide" Gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

The raw sequence data obtained were then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231) version 97-0. The individual sequences of the pZero1 derivatives were assembled to a continuous contig. The computer-assisted coding region analysis was prepared with the XNIP program (Staden, 1986, Nucleic Acids Research, 14:217-231).

15 The resulting nucleotide sequence is shown in SEQ ID No. 1. Analysis of the nucleotide sequence showed an open reading frame of 1313 base pairs, which was called the metY gene. The metY gene codes for a protein of 437 amino acids.

Example 3

20 Construction of vectors for expression of metY and metAY

3.1. Amplification of the genes metY and metA

The methionine biosynthesis genes metA and metY from C. glutamicum were amplified using the polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the 25 nucleotide sequences of the methionine biosynthesis genes metY (SEQ ID No.1) and metA (gene library entry Accession Number AF052652) of C. glutamicum ATCC 13032, PCR primers were synthesized (MWG Biotech, Ebersberg, Germany). These primers were chosen so that the amplified fragments contain the genes 30 and native ribosome binding sites thereof, but not possible promoter regions. In addition, suitable restriction cutting sites which allow cloning into the target vector were inserted. The sequences of the PCR primers, the cleavage sites inserted (sequence underlined) and the amplified gene

(the fragment size, in bp, is listed in parentheses) are listed in the following Table 1.

Table 1

Primer	Sequence with restriction cleavage site	Product	Plasmid
metY-EVP5	5' - CTAATAAG <u>T</u> CGACAAAGGAGGACA Sall ACCATGCCAAAGTACGAC- 3'	metY (1341 bp)	pCREmetY
metY-EVP3	5' - GAGTCTAAT <u>G</u> CATGCTAGATTGCA NsiI GCAAAGCCG 3'		
meta-EVP5	5' - AGAAC <u>G</u> AATTCAAAGGAGGACAAC EcoRI CATGCCAAC <u>C</u> TCGCGC- 3'	meta (1161 bp)	pCREmeta
meta-EVP3	5' - GTCGTGGAT <u>CC</u> CTATTAGATGTA PstI GAAC <u>T</u> CG- 3'		

5 The PCR experiments were carried out with the Taq DNA polymerase from Gibco-BRL (Eggestein, Germany) in a "PCT-100 Thermocycler" (MJ Research Inc., Watertown, Mass., USA). A single denaturing step of 2 minutes at 94°C was followed by a denaturing step of 90 seconds (sec) at 94°C, an annealing step 10 for 90 sec at a primer-dependent temperature of $T = (2xAT + 4xGC) - 5$ C (Suggs, et al., 1981, p. 683-693, In: D.D. Brown, and C.F. Fox (Eds.), Developmental Biology using Purified Genes. Academic Press, New York, USA) and an extension step at 72°C lasting 90 sec. The last three steps were repeated as a cycle 15 35 times and the reaction was ended with a final extension step of 10 minutes (min) at 72°C. The products amplified in this way were tested electrophoretically in a 0.8% agarose gel.

The metY fragment 1341 bp in size was cleaved with the 20 restriction endonucleases Sall and NsiI, and the meta fragment 1161 bp in size was cleaved with the restriction endonucleases EcoRI and BamHI. The two batches were separated by gel

electrophoresis and the fragments metY (approx. 1330 bp) and metA (approx. 1150 bp) were isolated from the agarose gel with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

5 3.2. Cloning of metY in the vector pZ8-1

The E. coli - C. glutamicum shuttle expression vector pZ8-1 (EP 0 375 889) was employed as the base vector for expression both in C. glutamicum and in E. coli. DNA of this plasmid was cleaved completely with the restriction enzymes SalI and PstI
10 and then dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250). The metY fragment isolated from the agarose gel in example 3.1 was mixed with the vector pZ8-1 prepared in this way and the batch was
15 treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no. 27-0870-04).

The ligation batch was transformed in the E. coli strain DH5 α (Hanahan, In: DNA cloning. A Practical Approach. Vol. I. IRL-
20 Press, Oxford, Washington DC, USA). Selection of plasmid-carrying cells was made by plating out the transformation batch on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l kanamycin. After incubation overnight at 37°C, recombinant individual clones were selected. Plasmid DNA was isolated
25 from a transformant with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and checked by restriction cleavage. The resulting plasmid was called pCREmetY. It is shown in figure 1.

30 3.3. Cloning of metA and metY in the vector pZ8-1

DNA of the plasmid pZ8-1 was cleaved completely with the restriction enzymes EcoRI and BamHI and then dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No.
35 1758250). The metA fragment isolated from the agarose gel in

example 3.1 was mixed with the vector pZ8-1 prepared in this way and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04) .

5 The ligation batch was transformed in the E. coli strain DH5 α (Hanahan, In: DNA cloning. A Practical Approach. Vol. I. IRL-Press, Oxford, Washington DC, USA). Selection of plasmid-carrying cells was made by plating out the transformation batch on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l kanamycin. After incubation overnight at 37°C, recombinant individual clones were selected. Plasmid DNA was isolated from a transformant with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and checked by

10 restriction cleavage. The resulting plasmid was called

15 pCREmetA.

The plasmid pCREmetA was cleaved completely with the restriction enzymes SalI and PstI and then dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 20 1758250). The metY fragment isolated from the agarose gel in example 3.1 was mixed with the vector pCREmetA prepared in this way and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description 25 T4-DNA-Ligase, Code no.27-0870-04) .

The ligation batch was transformed in the E. coli strain DH5 α (Hanahan, In: DNA cloning. A Practical Approach. Vol. I. IRL-Press, Oxford, Washington DC, USA). Selection of plasmid-carrying cells was made by plating out the transformation 30 batch on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l kanamycin. After incubation overnight at 37°C, recombinant individual clones were selected. Plasmid DNA was isolated from a transformant with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance 35 with the manufacturer's instructions and checked by

restriction cleavage. The resulting plasmid was called pCREmetAY. It is shown in figure 2.

Example 4

Preparation of the strains DSM5715/pCREmetY and
5 DSM5715/pCREmetAY

The vectors pCREmetY and pCREmetAY mentioned in example 3.2 and 3.3 were electroporated by the electroporation method of Tauch et al. (1994, FEMS Microbiological Letters, 123:343-347) in *Corynebacterium glutamicum* DSM 5715. The strain DSM 5715
10 is an AEC-resistant lysine producer. Selection for plasmid-carrying cells was made by plating out the electroporation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), which had been
15 supplemented with 25 mg/l kanamycin. Plasmid DNA was isolated in each case from a transformant by conventional methods (Peters-Wendisch et al., 1998, Microbiology 144, 915-927) and checked by restriction cleavage with subsequent agarose gel electrophoresis. The strains were called DSM5715/pCREmetY and
20 DSM5715pCREmetAY. The strain DSM5715/pCREmetY has been deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty as DSM 13556.

25 Example 5

Preparation of lysine with the strain DSM5715/pCREmetY

The *C. glutamicum* strain DSM5715/pCREmetY obtained in example 4 was cultured in a nutrient medium suitable for the production of lysine and the lysine content in the culture
30 supernatant was determined.

For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with kanamycin (50 mg/l)) for 24 hours at 33°C. Starting from this agar plate culture, a pre-culture was seeded (10 ml medium in a 100

ml conical flask). The complete medium CgIII was used as the medium for the pre-culture.

Medium Cg III

NaCl 2.5 g/l

Bacto-Peptone 10 g/l

Bacto-Yeast extract 10 g/l

Glucose (autoclaved separately) 2% (w/v)

The pH was brought to pH 7.4

Kanamycin (50 mg/l) was added to this. The pre-culture was
5 incubated for 16 hours at 33°C at 240 rpm on a shaking
machine. A main culture was seeded from this pre-culture such
that the initial OD (660 nm) of the main culture was 0.1.
Medium MM was used for the main culture.

Medium MM

CSL (corn steep liquor)	5 g/l
MOPS (morpholinopropanesulfonic acid)	20 g/l
Glucose (autoclaved separately)	50 g/l
$(\text{NH}_4)_2\text{SO}_4$	25 g/l
KH_2PO_4	0.1 g/l
$\text{MgSO}_4 \cdot 7 \text{ H}_2\text{O}$	1.0 g/l
$\text{CaCl}_2 \cdot 2 \text{ H}_2\text{O}$	10 mg/l
$\text{FeSO}_4 \cdot 7 \text{ H}_2\text{O}$	10 mg/l
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	5.0mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
L-Leucine (sterile-filtered)	0.1 g/l
CaCO_3	25 g/l

The CSL, MOPS and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the CaCO_3
5 autoclaved in the dry state.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Kanamycin (50 mg/l) was added. Culturing was carried out at 33°C and 80% atmospheric humidity.

After 48 hours, the OD was determined at a measurement
10 wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of lysine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivation with ninhydrin detection.

The result of the experiment is shown in Table 2.

Table 2

Strain	OD(660)	Lysine HCl g/l
DSM5715	10.6	15.7
DSM5715/pCREmetY	9.5	16.1

Example 6

5 Preparation of methionine with the strain DSM5715/pCREmetAY

The C. glutamicum strain DSM5715/pCREmetAY obtained in example 4 was cultured in a nutrient medium suitable for the production of methionine and the methionine content in the culture supernatant was determined.

10 For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with kanamycin (50 mg/l)) for 24 hours at 33°C. Starting from this agar plate culture, a pre-culture was seeded (10 ml medium in a 100 ml conical flask). The complete medium CgIII as described in 15 example 5 was used as the medium for the pre-culture.

Kanamycin (50 mg/l) was added to this. The pre-culture was incubated for 16 hours at 33°C at 240 rpm on a shaking machine. A main culture was seeded from this pre-culture such that the initial OD (660 nm) of the main culture was 0.1. The 20 medium MM as described in example 5 was used for the main culture.

The CSL, MOPS and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the CaCO₃, 25 autoclaved in the dry state.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Kanamycin (50 mg/l) was added. Culturing was carried out at 33°C and 80% atmospheric humidity.

After 72 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of methionine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivation with ninhydrin detection.

The result of the experiment is shown in Table 3.

Table 3

Strain	OD(660)	Methionine g/l
DSM5715	6.6	1.4
DSM5715/pCREmetAY	8.3	16.0

Brief Description of the Figures:

Figure 1: Plasmid pCREmetY

Figure 2: Plasmid pCREmetAY

The abbreviations used in the figures have the following meaning:

Kan: Resistance gene for kanamycin

metY: metY gene of *C. glutamicum*

metA: metA gene of *C. glutamicum*

Ptac: tac promoter

rrnB-T1T2: Terminator T1T2 of the rrnB gene of *E. coli*

rep: Plasmid-coded replication origin for
C. glutamicum (of pHM1519)

BamHI: Cleavage site of the restriction enzyme BamHI

EcoRI: Cleavage site of the restriction enzyme EcoRI

5 EcoRV: Cleavage site of the restriction enzyme EcoRV

PstI: Cleavage site of the restriction enzyme PstI

SalI: Cleavage site of the restriction enzyme SalI

XhoI: Cleavage site of the restriction enzyme XhoI

10 This disclosure is based on priority documents DE 100 43
334.0, DE 101 09 690.9 and U.S. 60/294,252, each incorporated
by reference.

Obviously, numerous modifications of the invention are
possible in view of the above teachings. Therefore, within
15 the scope of the appended claims, the invention may be
practiced otherwise than as specifically described herein.

SEQUENCE PROTOCOL

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15 <170> PatentIn Ver. 2.1

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gtctattgca atagaccaag ctgttcagta gggtgcattgg gagaagaatt tcctaataaa 180

aactcttaag gacctccaa atg cca aag tac gac aat tcc aat gct gac cag 232
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1

5

10

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15 20 25

40

cag acc agc gca cga aac ctt ccg atc tac caa tcc acc gct ttc gtg 328
Gln Thr Ser Ala Arg Asn Leu Pro Ile Tyr Gln Ser Thr Ala Phe Val
30 35 40

45

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Gly Pro Val Tyr Ser Arg Leu Thr Asn Pro Thr Val Glu Ala Leu Glu
60 65 70 75

55

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Asn Arg Ile Ala Ser Leu Glu Gly Val His Ala Val Ala Phe Ser
80 85 90

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	175 180 185	
30	ctg atc atc gac aac acc atc gct acc gca gcg ctc gtg cgc ccg ctc Leu Ile Ile Asp Asn Thr Ile Ala Thr Ala Ala Leu Val Arg Pro Leu	808
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5 aag tac acc ggc tcc gtt ctc acc ttc gag atc aag ggc ggc aag gat 1288
Lys Tyr Thr Gly Ser Val Leu Thr Phe Glu Ile Lys Gly Gly Lys Asp
350 355 360

10 gag gct tgg gca ttt atc gac gcc ctg aag cta cac tcc aac ctt gca 1336
Glu Ala Trp Ala Phe Ile Asp Ala Leu Lys Leu His Ser Asn Leu Ala
365 370 375

15 aac atc ggc gat gtt cgc tcc ctc gtt gtt cac cca gca acc acc acc 1384
Asn Ile Gly Asp Val Arg Ser Leu Val Val His Pro Ala Thr Thr Thr
380 385 390 395

20 cat tca cag tcc gac gaa gct ggc ctg gca cgc gcg ggc gtt acc cag 1432
His Ser Gln Ser Asp Glu Ala Gly Leu Ala Arg Ala Gly Val Thr Gln
400 405 410

tcc acc gtc cgc ctg tcc gtt ggc atc gag acc att gat gat atc atc 1480
Ser Thr Val Arg Leu Ser Val Gly Ile Glu Thr Ile Asp Asp Ile Ile
415 420 425

25 gct gac ctc gaa ggc ggc ttt gct gca atc tagcttaaaa tagactcacc 1530
Ala Asp Leu Glu Gly Gly Phe Ala Ala Ile
430 435

30 ccagtgccta aagcgctggg tttttctttt tcagactcgt gagaatgcaa actagactag 1590

acagagctgt ccatatacac tggacgaagt tttagtcttg tccacccaga acaggcggtt 1650

attttcatgc ccaccctcgc gccttcaggt caacttgaaa tccaagcgat cggtgatgtc 1710

35 tccaccgaag 1720

40 <210> 2
<211> 437
<212> PRT
<213> Corynebacterium glutamicum

45 Met Pro Lys Tyr Asp Asn Ser Asn Ala Asp Gln Trp Gly Phe Glu Thr
1 5 10 15

Arg Ser Ile His Ala Gly Gln Ser Val Asp Ala Gln Thr Ser Ala Arg
20 25 30

50 Asn Leu Pro Ile Tyr Gln Ser Thr Ala Phe Val Phe Asp Ser Ala Glu
35 40 45

His Ala Lys Gln Arg Phe Ala Leu Glu Asp Leu Gly Pro Val Tyr Ser
50 55 60

Arg Leu Thr Asn Pro Thr Val Glu Ala Leu Glu Asn Arg Ile Ala Ser
65 70 75 80

60 Leu Glu Gly Gly Val His Ala Val Ala Phe Ser Ser Gly Gln Ala Ala
85 90 95

Thr Thr Asn Ala Ile Leu Asn Leu Ala Gly Ala Gly Asp His Ile Val
 100 105 110

5 Thr Ser Pro Arg Leu Tyr Gly Gly Thr Glu Thr Leu Phe Leu Ile Thr
 115 120 125

Leu Asn Arg Leu Gly Ile Asp Val Ser Phe Val Glu Asn Pro Asp Asp
 130 135 140

10 Pro Glu Ser Trp Gln Ala Ala Val Gln Pro Asn Thr Lys Ala Phe Phe
 145 150 155 160

Gly Glu Thr Phe Ala Asn Pro Gln Ala Asp Val Leu Asp Ile Pro Ala
 15 165 170 175

Val Ala Glu Val Ala His Arg Asn Ser Val Pro Leu Ile Ile Asp Asn
 180 185 190

20 Thr Ile Ala Thr Ala Ala Leu Val Arg Pro Leu Glu Leu Gly Ala Asp
 195 200 205

Val Val Val Ala Ser Leu Thr Lys Phe Tyr Thr Gly Asn Gly Ser Gly
 210 215 220

25 Leu Gly Gly Val Leu Ile Asp Gly Gly Lys Phe Asp Trp Thr Val Glu
 225 230 235 240

Lys Asp Gly Lys Pro Val Phe Pro Tyr Phe Val Thr Pro Asp Ala Ala
 30 245 250 255

Tyr His Gly Leu Lys Tyr Ala Asp Leu Gly Ala Pro Ala Phe Gly Leu
 260 265 270

35 Lys Val Arg Val Gly Leu Leu Arg Asp Thr Gly Ser Thr Leu Ser Ala
 275 280 285

Phe Asn Ala Trp Ala Ala Val Gln Gly Ile Asp Thr Leu Ser Leu Arg
 290 295 300

40 Leu Glu Arg His Asn Glu Asn Ala Ile Lys Val Ala Glu Phe Leu Asn
 305 310 315 320

Asn His Glu Lys Val Glu Lys Val Asn Phe Ala Gly Leu Lys Asp Ser
 45 325 330 335

Pro Trp Tyr Ala Thr Lys Glu Lys Leu Gly Leu Lys Tyr Thr Gly Ser
 340 345 350

50 Val Leu Thr Phe Glu Ile Lys Gly Gly Lys Asp Glu Ala Trp Ala Phe
 355 360 365

Ile Asp Ala Leu Lys Leu His Ser Asn Leu Ala Asn Ile Gly Asp Val
 370 375 380

55 Arg Ser Leu Val Val His Pro Ala Thr Thr His Ser Gln Ser Asp
 385 390 395 400

Glu Ala Gly Leu Ala Arg Ala Gly Val Thr Gln Ser Thr Val Arg Leu
 60 405 410 415

Ser Val Gly Ile Glu Thr Ile Asp Asp Ile Ile Ala Asp Leu Glu Gly

420

425

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Gly Phe Ala Ala Ile

5

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Claims:

1. An isolated polynucleotide comprising a polynucleotide sequence which codes for the metY gene of coryneform bacteria, selected from the group consisting of
 - 5 a) polynucleotide which is at least 70% identical to a polynucleotide that codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,
 - b) polynucleotide which codes for a polypeptide that comprises an amino acid sequence which is at least 70% identical to the amino acid sequence of SEQ ID No. 2,
 - 10 c) polynucleotide which is complementary to the polynucleotides of a) or b), and
 - d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c).
2. The polynucleotide of claim 1, which is capable of replication in coryneform bacteria.
3. The polynucleotide of claim 1, wherein the polynucleotide is an RNA.
4. The polynucleotide of claim 2, comprising the nucleic acid sequence of SEQ ID No. 1.
5. The DNA of claim 2 which is capable of replication, comprising
 - 25 (i) the nucleotide sequence shown in SEQ ID No. 1, or
 - (ii) at least one sequence which corresponds to sequence (i) within the range of the degeneration of the genetic code, or

(iii) at least one sequence which hybridizes with a sequence complementary to sequence (i) or (ii), and optionally

(iv) sense mutations of (i).

5 6. The DNA of claim 5 which is capable of replication, wherein the hybridization of sequence (iii) occurs at a stringency corresponding to at most 2x SSC.

7. A polynucleotide sequence of claim 2, which codes for a polypeptide which comprises the amino acid sequence in
10 SEQ ID No. 2.

8. Corynebacterium glutamicum strain DSM5715/pCREmetY as DSM 13556 deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures), Braunschweig, Germany.

15 9. A process for the fermentative preparation of L-amino acids, comprising:

- a) fermentation of the coryneform bacteria which produce the desired amino acid and in which at least the metY gene or nucleotide sequences which code for it are enhanced;
- b) concentration of the L-amino acid in the medium or in the cells of the bacteria; and
- c) isolation of the L-amino acid.

20 10. A process for the fermentative preparation of L-methionine, comprising:

- a) fermentation of an L-methionine-producing coryneform bacteria in which the metY gene, optionally with met A, is enhanced;
- b) concentration of said L-amino acid in the medium or in the cells of the bacteria; and

c) isolation of said L-amino acid.

11. The process of claim 9 or 10, wherein bacteria in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced are employed.

5 12. The process of claim 9 or 10, wherein bacteria in which the metabolic pathways which reduce the formation of the desired amino acid are at least partly eliminated are employed.

10 13. The process of claim 9, wherein a strain transformed with a plasmid vector is employed, and the plasmid vector carries the metY gene and optionally additionally the metA gene.

15 14. The process of claim 10, wherein a strain transformed with a plasmid vector is employed, and the plasmid vector carries the nucleotide sequence which codes for the metA and metY genes.

15. The process of claim 9, wherein for the preparation of L-lysine, the coryneform microorganisms have one or more enhanced genes selected from the group consisting of

20 15.1 gap gene which codes for glycerolaldehyde 3-phosphate dehydrogenase,

15.2 tpi gene which codes for triose phosphate isomerase,

25 15.3 pgk gene which codes for 3-phosphoglycerate kinase,

15.4 pyc gene which codes for pyruvate carboxylase,

15.5 lysC gene which codes for a feed back resistant aspartate kinase.

30 16. The process of claim 10, wherein the coryneform microorganisms have one or more enhanced genes selected from the group consisting of

16.1 the lysC gene which codes for a feed back resistant aspartate kinase,

16.2 the gap gene which codes for glycerolaldehyde 3-phosphate dehydrogenase,

5 16.3 the tpi gene which codes for triose phosphate isomerase,

16.4 the metA gene which codes for homoserine O-acetyltransferase,

10 16.5 the metB gene which codes for cystathionine-gamma-synthase,

16.6 the aecD gene which codes for cystathionine-gamma-lyase,

16.7 the glyA gene which codes for serine hydroxymethyltransferase

15 16.8 the pgk gene which codes for 3-phosphoglycerate kinase

16.9 the pyc gene which codes for pyruvate carboxylase.

17. The process of claim 16, wherein the coryneform microorganisms have an additional enhancement of the metY gene by metA.

20 18. The process of claim 9, wherein the coryneform microorganisms have an additional enhancement of the metY gene by attenuation of one or more genes selected from the group consisting of

25 18.1 the pck gene which codes for phosphoenol pyruvate carboxykinase

18.2 the pgi gene which codes for glucose 6-phosphate isomerase

18.3 the poxB gene which codes for pyruvate oxidase.

19. The process of claim 10, wherein the coryneform microorganisms have one or more attenuated genes selected from the group consisting of
- 19.1 the thrB gene which codes for homoserine kinase
- 5 19.2 the ilvA gene which codes for threonine dehydratase
- 19.3 the thrC gene which codes for threonine synthase
- 19.4 the ddh gene which codes for meso-diaminopimelate D-dehydrogenase
- 19.5 the pck gene which codes for phosphoenol pyruvate carboxykinase
- 10 19.6 the pgi gene which codes for glucose 6-phosphate isomerase
- 19.7 the poxB gene which codes for pyruvate oxidase.
20. A coryneform bacterium in which the metY gene is enhanced.
- 15 21. A coryneform bacterium that contains a vector which carries a polynucleotide of claim 1.
22. The process of claims 9 or 10, wherein microorganisms of the species *Corynebacterium glutamicum* are employed.
- 20 23. The process of claim 22, wherein the *Corynebacterium glutamicum* strain DSM5715/pCREmetY is employed.
24. The process of claim 22, wherein the *Corynebacterium glutamicum* strain DSM5715/pCREmetAY is employed.
25. A process for preparing an L-methionine-containing animal feedstuffs additive comprising:
- a) culture and fermentation of an L-methionine-producing microorganism in a fermentation medium;

- b) removal of water from the L-methionine-containing fermentation broth (concentration);
 - c) removal of an amount of 0 to 100 wt.% of the biomass formed during the fermentation; and
 - 5 d) drying of the fermentation broth obtained according to b) and/or c) to obtain the animal feedstuffs additive in the desired powder or granule form.
26. The process of claim 25, wherein microorganisms are employed in which further genes of the biosynthesis pathway of L-methionine are additionally enhanced.
- 10 27. The process of claim 26, wherein microorganisms are employed in which the metabolic pathways which reduce the formation of L-methionine are at least partly eliminated.
28. The process of claim 25, wherein expression of the polynucleotides which code for the metY gene is enhanced.
- 15 29. The process of claim 25, wherein microorganisms of the species *Corynebacterium glutamicum* are employed.
30. The process of claim 29, wherein the *Corynebacterium glutamicum* strain DSM5715/pCREmetY is employed.
- 20 31. The process of claim 29, wherein the *Corynebacterium glutamicum* strain DSM5715/pCREmetAY is employed.
32. The process of claim 25, wherein one or more of the following steps are additionally carried out:
- 25 e) addition of one or more organic substances, including L-methionine and/or D-methionine and/or the racemic mixture D,L-methionine, to the products obtained according to b), c) and/or d);
 - f) addition of auxiliary substances selected from the group consisting of silicas, silicates, stearates, grits and bran to the substances obtained according

to b) to e) for stabilization and to increase storability; or

g) conversion of the substances obtained according to b) to f) into a form which remains stable in rumen, 5 by coating them with film-forming agents.

33. The process of claim 25 or 32, wherein some of the biomass is removed.

34. The process of claim 33, wherein essentially 100% of the biomass is removed.

10 35. The process of claim 25 or 32, wherein the water content is up to 5 wt.%.

36. The process of claim 35, wherein the water content is less than 2 wt.%.

15 37. The process of claim 32, wherein the film-forming agents are metal carbonates, silicas, silicates, alginates, stearates, starches, gums or cellulose ethers.

38. An animal feedstuffs additive prepared as claimed in claim 25.

20 39. An animal feedstuffs additive as claimed in claim 38, which comprises 1 wt.% to 80 wt.% L-methionine, D-methionine, D,L-methionine or a mixture thereof, based on the dry weight of the animal feedstuffs additive.

25 40. A process for obtaining RNA, cDNA or DNA in order to isolate nucleic acids, or polynucleotides or genes which code for O-acetylhomoserine sulphhydrolase or which have a high similarity to the sequence of the metY gene, which comprises employing polynucleotides of claim 1 as hybridization probes.

Abstract

An isolated polynucleotide comprising a polynucleotide sequence selected from the group consisting of

- a) polynucleotide which is at least 70% identical to a
5 polynucleotide that codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,
- b) polynucleotide which codes for a polypeptide that comprises an amino acid sequence which is at least 70% identical to the amino acid sequence of SEQ ID No. 2,
- 10 c) polynucleotide which is complementary to the polynucleotides of a) or b), and
- d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),
and a process for the fermentative preparation of L-amino
15 acids using coryneform bacteria in which at least the metY gene is present in enhanced form, and use of the polynucleotides as hybridization probes.